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(54) Title: PROCEDURE FOR THE AMPLIFICATION OF AT LEAST ONE SPECIFIC NUCLEOTIDE SEQUENCE, AND IMPLEMENTATION PRIMERS

(57) Abstract

[please see the existing English-language abstract in the original French document]

Procedure for the amplification of at least one specific nucleotide sequence, and implementation primers

The present invention relates to a new procedure for the amplification of at least one specific nucleotide sequence of a synthetic or natural nucleic acid contained within a reaction mixture. The invention also relates to primers that enable the implementation of such an amplification.

The state of the art describes methods that allow the amplification of nucleotide sequences using primers that are specific for the sequences to be amplified. Accordingly, a gene or a family of genes can be amplified within a preparation consisting of nucleic acids. Many methods use oligonucleotides that are complementary to the target sequence and that serve as primers for elongation via a polymerase.

DNA [deoxyribonucleic acid] can be amplified via PCR (the Polymerase Chain Reaction), as described in U.S. patents No. 4,683,195, No. 4,683,202, and No. 4,800,159; via LCR (the Ligase Chain Reaction), as described, for example, in European patent application No. 0,201,184; and via RCR (the Repair Chain Reaction), as described in [PCT] patent application No. WO 90/01069.

Several methods for the amplification of RNA [ribonucleic acid] have also been described in various documents. These methods are listed below:

- *3SR (self-sustained sequence replication), as described in [PCT] patent application No. WO 90/06995;*

- *NASBA (nucleic-acid sequence-based amplification), as described in [PCT] patent application No. WO 91/02818;*

- *SPSR (single primer sequence replication), as described in U.S. patent No. 5,194,370; and*

- *TMA (transcription-mediated amplification), as described in U.S. patent No. 5,399,491.*

Nevertheless, these methods impose a strict selection of amplification primers. In fact, primers that are not specific for the desired sequence will allow the amplification of numerous related sequences on the sequence to which they are affixed. As a result, the amplicon that corresponds to the desired sequence is diluted within a mixture of amplicons, thus making the amplification product difficult to use. Under these conditions, therefore, strict selection is imposed, such that a region of the nucleotide sequence is chosen that is sufficiently specific to allow the production of a complementary primer with comparable specificity.

However, other problems may arise in connection with the choice of the sequence to be used for the hybridization of the primer. In fact, as it happens, the truly specific region (which is unique) is located within the desired sequence. When a primer associated with such a region is hybridized, only a more-or-less sizable fraction of the desired sequence is obtained, and thus information is lost. The procedure for obtaining a primer that is specific for the desired nucleotide sequence also entails a major increase in difficulty and in labor.

The present invention obviates the risks of obtaining truncated amplification products and the difficulties associated with obtaining primers that are specific for the nucleotide sequence to be amplified, because the invention enables the specific amplification of the desired nucleotide sequence under known traditional hybridization conditions.

To obtain the said amplification, two types of complementary primers are used, i.e., on the one hand, a primer that hybridizes indiscriminately with all of the related nucleotide sequences and, on the other hand, a primer in which each primer hybridizes with only one of the related nucleotide sequences. The primers of the first type, which are non-specific, are used as elongation primers, and the primers of the second type, which are specific for the nucleotide

sequences that are related to the desired sequence, block the elongation of some of the said related nucleotide sequences.

When a mixture of non-specific and specific primers is used, the elongation of certain non-specific sequences can be inhibited, in accordance with the type of specific sequences utilized. Thus, the amplicons that will be obtained can be chosen beforehand.

Accordingly, the amplification of certain related sequences that one does not wish to amplify can be blocked, through the addition of complementary sequences that are specific for these related sequences, such that these specific complementary sequences serve as blocking primers. Thus, the desired sequence(s) that will be selectively amplified can be isolated, and a single amplicon is obtained for each desired sequence for which no blocking primer was added.

Accordingly, the present invention relates to a procedure for the amplification of at least one specific nucleotide sequence of a synthetic or natural nucleic acid contained within a reaction mixture, with the said reaction mixture consisting of at least one nucleic acid that has at least two related nucleotide sequences and/or at least two nucleic acids, each of which includes at least one related nucleotide sequence. The said procedure utilizes at least one type of amplification primer that is capable of hybridizing with nucleic acid in order to allow the amplification of related nucleotide sequences, and is characterized in that the said procedure consists of adding, to the reaction mixture, at least one sequence, which serves as a blocking primer, which is able to:

- Hybridize with at least one nucleotide sequence that is not the specific nucleotide sequence or sequences to be amplified; and

- Inhibit, in its immediate region, the elongation of the amplification trigger.

The blocking primer or primers are preferably capable of hybridizing with the nucleotide sequence that is not the specific nucleotide sequence to be amplified, or to all such nucleotide sequences.

First of all, when a blocking primer is utilized in an amplification procedure as described here-in above, each blocking primer consists of an oligonucleotide that has a base of deoxyribo-nucleotides and/or ribonucleotides and/or modified nucleotides, such as PNAs or thiophosphate nucleotides.

Secondly, when a blocking primer is utilized in an amplification process as described herein-above, each blocking primer includes at least one element that prevents the said amplification.

The element that prevents the amplification is located at the 3' end of the blocking primer and does not allow its elongation.

Furthermore, another element that prevents amplification is located at the 5' end of the blocking primer, and serves as a protective element.

Each element that prevents amplification consists of:

- Either a nucleotide or a modified nucleotide, or an oligonucleotide that does or does not include a modified nucleotide, such that the nucleotide, the modified nucleotide, or the oligonucleotide does not hybridize with the nucleic acid; or

- A molecule that is different from a nucleotide or a modified nucleotide.

In this case, the said element consists of at least five, and in particular at least ten, and preferably at least fifteen nucleotides or modified nucleotides, or a mixture of nucleotide(s) and modified nucleotide(s).

In a first embodiment of the invention, in which the element consists of a nucleotide, a modified nucleotide, or an oligonucleotide that does or does not include a modified nucleotide, the said nucleotide, modified nucleotide, or oligonucleotide does not hybridize with the nucleic acid, and

the said element is long enough to allow the formation of a loop, with hybridization between the nucleotides and/or the modified nucleotides that constitute the said loop.

In a second embodiment of the invention, in which the said element consists of a nucleotide, a modified nucleotide, or an oligonucleotide that does or does not include a modified nucleotide, the said nucleotide, modified nucleotide, or oligonucleotide does not hybridize with the nucleic acid, and the said element consists of a so-called "tail" of polynucleotides and/or modified polynucleotides, which all include the same bases.

When a blocking primer is utilized in an amplification process that includes an element that does not allow elongation, the element is substituted at the hydrogen atom of the hydroxyl group or at the hydroxyl group itself, located at position 3' on the ribose, which itself is located at the 3' end of the nucleic acid.

When a blocking primer is utilized in an amplification process that also includes a protective element, the said element is:

- Substituted at the phosphate located at position 5' on the ribose, which itself is located at the 5' end of the nucleic acid; or

- Grafted onto the phosphate located at position 5' on the ribose, which itself is located at the 5' end of the nucleic acid.

The attached figures are provided as an illustrative example, and are in no way limitative.

Figure 1 is a diagrammatic view of the principle underlying the amplification of a strand of nucleic acid and its complementary strand, by means of two primers. In this case, which is the simplest case, there is one primer per strand.

Figure 2 is a diagrammatic view of the principle underlying the amplification shown in Figure 1, but with the use of the technique disclosed in the present invention.

Figure 3 represents the various substitutions that may be performed on the nucleotides of the blocking primer, in which:

- R1 is an element that is located at the 3' end of the blocking primer and that prevents any elongation during amplification;

- R2 is an element that can be found in at least one of the 2' positions on the ribose of a nucleotide of the blocking primer, and that increases the stability of the duplex consisting of the blocking primer and the nucleic acid; and

- R3 is an element that is located at the 5' end of the blocking primer and that serves as a protective element.

Figure 4 represents the positioning of the R1 element at the 3' end of the blocking primer when the said primer is hybridized with the nucleic acid.

Figure 5 represents the positioning of the R3 element at the 5' end of the blocking primer when the said primer is hybridized with the nucleic acid.

Figure 6 represents the duplex consisting of the blocking primer and the nucleic acid, in which X represents a nucleotide of the blocking primer, which includes the R2 element that increases the stability of the duplex, in position on the ribose of this nucleotide.

Figure 7 represents various different structures which, when added at the 3' position on the blocking primer, prevent any elongation during amplification.

Figure 8 represents various different structures which, when added at the 5' position on the blocking primer as a complement to the modifications at position 3', as shown in Figure 3, serve as a protective element by preventing the degradation or ejection of the blocking primer during amplification.

Figure 9 represents the electrophoretogram that corresponds to the result of the reaction sequence of a sample of DNA from an HLA-DRB1*1301 and HLA-DRB3*01 lymphoblastoid line, as observed for the region that codes for amino acids 56 through 65 (according to the official HLA nomenclature) of the HLA-DRB genes without the use of a blocking primer.

Figure 10 represents the electrophoretogram that corresponds to the result of the sequence reaction of a DNA sample from an HLA-DRB1*1301 and HLA-DRB3*01 lymphoblastoid line, as observed for the region that codes for amino acids 56 through 65 (according to the official HLA nomenclature) of the HLA-DRB genes with the use of a 5'–phosphate / 3'–C_6–NH_2 oligonucleotide that inhibits the amplification of the HLA-DRB3 gene.

Figure 11 represents the electrophoretogram that corresponds to the result of the sequence reaction of a DNA sample from an HLA-DRB1*0901 and HLA-DRB4*01 lymphoblastoid line, as observed for the region that codes for amino acids 29 through 47 (according to the official HLA nomenclature) of the HLA-DRB genes without the use of a blocking primer.

Figure 12 represents the electrophoretogram that corresponds to the result of the sequence reaction of a sample of DNA from an HLA-DRB1*0901 and HLA-DRB4*01 lymphoblastoid line, as observed for the region that codes for amino acids 29 through 47 (according to the official HLA nomenclature) of the HLA-DRB genes, with the use of a 5'–acridine / 3'–H oligonucleotide that inhibits the amplification of the HLA-DRB4 gene.

Figure 13 is a diagrammatic view of the principle of selective amplification of a gene in a family of related genes located on the same chromosome.

Consequently, among other things, the present invention relates, to the use of oligonucleotide primers, which have been modified at their ends, for the selective amplification of genes.

The invention also relates to a method that utilizes modified oligonucleotide primers for the selective amplification of certain genes that are present in a group of related genes.

The analysis of genes of interest is facilitated by the use of genetic amplification techniques that make it possible to prepare, from a biological sample, quantities of specific material that can easily be analyzed via known traditional molecular biological techniques. Thus, the use of oligonucleotide primers that limit a given genetic region leads to the obtention of a mixture of nucleotidic molecules that is considerably enriched in the desired molecule, which is then easy to detect through electrophoretic analytical methods or molecular hybridization methods. The effectiveness of this approach lies in the use of oligonucleotide primers that are specific for the genetic regions to be analyzed. Consequently, these primers must consist of oligonucleotide sequences that are capable of hybridizing selectively with the desired nucleic sequences that are present in the sample.

Nevertheless, the analysis of the genes that are members of structurally similar families of genes can sometimes be a delicate matter. The detection of nucleotide regions that are unique for a given gene makes it possible to achieve the desired specificity, but this approach may sometimes be difficult, or even impossible.

Thus, the present invention consists of combining the use of oligonucleotide primers that are specific for the amplification of a limited group of structurally similar genes, and the use of modified oligonucleotides that are capable of blocking specifically the undesirable genes. In point of fact, one single type of undesirable gene corresponds to each type of these modified primers.

This strategy makes it possible to simplify the analysis of genes in a mixture, through the determination of their nucleotide sequence (through gel sequencing, for example, or through multiple DNA chip hybridization) or through the analysis of mutations.

The present invention claims the use of mixtures consisting of nucleotide primers, which enable the effective amplification of corresponding nucleic regions, and blocking nucleotide triggers, either overlapping or located downstream (in relation to the nucleotide primer that enables the

amplification), which consist of oligonucleotides that cannot serve as initiating sequences for elongation or, consequently, for the amplification of the downstream sequences.

Therefore, for an undesirable gene or allele, the non-blocking primer and the blocking primer hybridize with the same strand for a given primer polarity (5' primers upstream, or 3' primers downstream, of the region to be analyzed). If it successfully hybridizes with the strand to be amplified, the non-blocking primer cannot generate amplicons beyond the region corresponding to the hybridization site of the blocking primer. Consequently, the amplification corresponding to the non-blocking primer is nullified. This principle, which is illustrated in figures 1, 2, and 13, relates to the functioning of the blocking primers and of the non-blocking primers.

As shown in Figure 1, amplification is performed with non-blocking primers P1 and P2. The extension of the P1 and P2 primers takes place in an altogether conventional way, and multiple amplicons A are obtained.

As shown in Figure 2, the procedure illustrated in Figure 1 is repeated exactly, because it involves amplification with non-blocking primers P1 and P2. However, on the complementary strand and downstream of the progress of the elongation of the P1 primer, a sequence is added that serves as a blocking primer (P1b), which is capable of hybridizing to the complementary strand, and of preventing amplification in its immediately surrounding region. In this case, no amplicon will be produced.

The procedure illustrated in Figure 1 and Figure 2 is repeated exactly in Figure 13, because the procedure in question involves the selective amplification of the G2 gene through blocking of the related G1 and G3 genes, via the use of specific blocking primers and non-specific amplification primers.

The invention also claims the use of blocking primers that include modified nucleotides. This principle is illustrated in figures 3 through 6.

As shown in Figure 3, the nucleotide can be modified at positions 2' or 3' on the ribose, near the 3' end of the oligonucleotide, and at position 5' on the ribose, near the 5' end of the said oligonucleotide.

As shown in Figure 4, the R1 group replaces the hydroxyl at position 3' of the ribose and, when the duplex consisting of the nucleic acid and the blocking primer is formed, prevents the elongation of the 3' end of the primer by the polymerase.

As shown in Figure 5, the R3 group replaces the phosphate at the 5' position on the ribose and, during the elongation of the amplification primer, protects the blocking primer against the degradation of the 5' end and/or any displacement of the blocking primer,

As shown in Figure 6, the duplex consisting of the nucleic acid and the blocking primer may be strengthened through substitution of the hydroxyl or of the hydrogen at position 2' on the ribose, which substitution may be made on several nucleotides of the blocking primer. The R2 group may, for example, consist of a 2' O-methyl radical, which stabilizes the DNA-RNA duplex by creating a hydrophobic interaction.

The claimed strategy has multiple applications that can be implemented whenever a mixture of related sequences is to be analyzed – for example, in human or animal genetics or in the analysis of infectious agents (viruses, bacteria, parasites, etc.).

Examples consisting of applications in the field of HLA (Human Leukocyte Antigens) are described hereinbelow.

The Major Histocompatibility Complex (MHC) includes a set of genes located on chromosome 6 in humans, [which genes are] involved in the regulation of the immune response (see Bodmer et al., *Nomenclature for Factors of the HLA System*, 1996; [and] *Tissue Antigens*, 1997, Vol. 49, pp. 297-321). These display extensive polymorphism, along with a very specific set of versions (or alleles) of each of these genes for each individual. It should be noted that each individual has two alleles of each gene, one of which is inherited from the mother and the other from the father.

Some of these MHC genes (which are more commonly referred to as the “HLA genes”) are already well known, in terms of both their nucleic sequence and the functions of the corresponding proteins. These genes consist essentially of the so-called “Class I” HLA genes (HLA-A, HLA-B, and HLA-Cw) and the so-called “Class II” HLA genes (HLA-DR, HLA-DQ, and HLA-DP). These genes participate in the regulation of the immune response, in terms of monitoring the integrity of the organism, with varying consequences in the medical field.

A first application relates to the field of organ or bone-marrow transplants, in which numerous studies have demonstrated the importance of optimal pairing between the organ donor and the recipient, in terms of the HLA genes involved in histocompatibility issues.

A second application relates to the study of the susceptibility of individuals to the development of certain pathologies induced by infectious agents (viruses, bacteria, and/or parasites) or by other mechanisms that are still not well known (as is the case, for example, with autoimmune diseases).

Thus, the HLA genes participate in the development of the very broad diversity of the immune response, as observed in each individual, for a given ethnic group. Lastly, the determination of the alleles of the HLA genes enables a precise characterization or identification of any individual, thereby constituting a third field of applicability for HLA typing.

One of the principal difficulties of HLA typing resides in the structural homology that has been observed for these genes, which have evolved from common ancestors. The result is that the genes of interest are analyzed within the context of similar functional genes or non-functional genes (i.e., pseudogenes). Consequently, it is essential that technique(s) for targeting the analysis of the nucleic sequences be mastered to the greatest extent possible, by optimizing the methods for the amplification of the regions to be sequenced.

For example, HLA-A typing is based on the selective analysis of the two alleles of the HLA-A gene observed in an individual, while avoiding the analysis of the structurally similar HLA-B

and HLA-Cw genes. Thus, it is essential to be able to amplify, specifically, the related regions observed for the HLA-A gene, by using nucleotide primers that are capable of hybridizing uniquely with the targeted regions on this gene.

Another example relates to HLA-DR typing, in which the analysis of polymorphism involves only the HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5 genes (which correspond to the genes that code for the polypeptide chains that constitute the functional proteins expressed on the surface of cells), by avoiding the co-amplification and analysis of the HLA-DRB2, HLA-DRB6, HLA-DRB7, and HLA-DRB9 pseudogenes. The process of HLA-DR typing illustrates the great complexity of the mixture of nucleic sequences to be identified in any given sample. The presence of two alleles for each of the genes further increases the difficulty, sometimes making the interpretation of the results very delicate (because of so-called "typing ambiguities").

With regard, for example, to HLA-DR typing, it can be asserted that the simplification of the analysis can be highly beneficial by restricting the analysis to the analysis of the HLA-DRB1 gene (with its two alleles for each individual), if the molecular analysis methods employed rely on the determination of signal intensity (i.e., the intensity of the fluorescence of molecules of increasing size as utilized in the sequencing), or, for example, on the interpretation of oligoprobe hybridization reactivity profiles. This goal can be achieved through the selection of nucleotide amplification primers that are specific for HLA-DRB1. However, this approach is not always possible, because of the nucleic sequences that are observed for the different alleles of the HLA-DRB1 gene, and due to the fact that sequences of other HLA-DRB genes share a high degree of homology.

The present invention provides an alternative, based on the use of a mixture of (a) primers that are specific for the HLA-DRB genes but non-specific for the HLA-DRB1 gene, and (b) blocking primers that are specific for the HLA-DRB3, HLA-DRB4, and HLA-DRB5 genes. Therefore, the result consists of selective amplification of the HLA-DRB1 genes, thereby enabling easier determination of the two HLA-DRB1 alleles, as observed for a given individual.

The nucleotide primers are synthesized according to traditional methods, such as (for example) the methods that use solid-phase synthesis, and, unless otherwise indicated, that include an –OH residue at 3' on the sugar (3'–OH) that enables their elongation during the amplification stage. The oligonucleotides employed consist essentially of oligonucleotides whose length is between 10 and 30 mers, depending on the application, which in turn depends on the nucleic sequences in question.

The blocking nucleotide primers, as prepared in accordance with the methods described hereinabove, contain a functional group, which inhibits elongation, located at the 3' end of the oligonucleotide. The purpose of this blocking function is to prevent the addition, by the DNA polymerase, of the following base, according to the information that is read on the complementary sequence. As an example, this functional blocking group at 3' may consist of a phosphate-alkylamine group (C_6-NH_2), phosphate, or dabcyl (see Figure 7). These groups protect the hydroxyl function (3'-OH), thereby blocking its reactivity during the polynucleotide polymerization catalyzed by the DNA polymerase. This blocking of the enzymatic polymerization can also be obtained through dehydroxylation of the 3' position. In point of fact, primers that contain 3'-H, 2'-OH ends can be obtained by using appropriate reagents in conjunction with the method consisting of oligonucleotide assembly on a solid substrate.

If circumstances call for the use of a blocking primer that does not overlap with the non-blocking primer, it may be advantageous to protect the 5' end of the blocking primer as well, so that the primer is neither degraded by the exonuclease activity of the polymerase, nor displaced during the elongation of the non-blocking primer located farther upstream (that is, toward the 5' end) of the region to be amplified. For this purpose, various modifications may make it possible to maintain the integrity of the hybridized blocking primer on the nucleic sequence destined to be inactivated. Several options can be cited as examples, e.g., the acridine core, dimethoxytrityl (DMT), the thiophosphate group, and an additional "stem-loop" sequence. Such modifications are amply illustrated in Figure 8.

The acridine core is a powerful intercalary agent that confers a very high degree of stability upon the duplex consisting of the primer and the target sequence and prevents the displacement of the primer. Dimethoxytrityl (DMT) uses, as a protective group for the 5'-hydroxyl end, the thio-phosphate group utilized in the anti-sense strategy, along with an additional sequence capable of forming a secondary "stem-loop" sequence that protects the primer from any potential degradation by an exonuclease activity.

Modification at the 3' end	C6–NH2 Phosphate H Dabcyl
Modification at the 5' end	Acridine DMT Thiophosphate The "stem-loop" structure

Table 1. – Modifications of the 3' (and) 5' ends of the blocking primers.

The principle of the use of mixtures of non-blocking and blocking primers may be applied for the 3' (downstream) end alone, or for both the 3' end and the 5' end (upstream of the region to be analyzed), depending on the characteristics of the nucleic sequences or the complexity of the genes in the region to be analyzed.

This amplification approach, which involves the use of blocking primers, may also be applied in simple amplification strategies associated with a mixture of primers that can hybridize with a given nucleic sequence to be analyzed, or in multiplex strategies, in which several mixtures of primers are utilized that can hybridize, during a single amplification reaction, with different nucleic sequences to be analyzed (e.g., with different loci, different genes, or different regions on a gene).

Example of the synthesis of oligonucleotide primers that are blocked at the 3' end and also at the 5' end

The blocked oligonucleotides primers were synthesized automatically on an Expidite (Perseptive *[sic]* Biosystems) device, in accordance with the phosphoramidite method, in accordance with the protocol proposed by the manufacturer. The phosphoramidite reagents required for the introduction of the modifications at the 3' and 5' ends were obtained from Glenn Research.

The oligonucleotides were purified via reverse-phase HPLC [high-performance liquid chromatography] (using a Beckman semi-preparative column, 25 cm long and with an ODS [outside diameter] of 10 mm; C18; 5 µm porosity; and a eluent with a gradient of 30 minutes containing 10% to 30% acetonitrile in a mixture with an aqueous solution of triethyl ammonium acetate (0.1M at a pH of 7)). The fractions containing the oligonucleotide were collected and dried, and the various different oligonucleotides were taken up in purified water and quantified via the measurement of UV [ultraviolet] absorption.

To illustrate the principle of the present invention, two examples of applications within the HLA field are described below.

<u>Example 1</u>: <u>Specific blocking of the amplification of the HLA-DRB3 gene</u>

Several HLA-DRB genes can code for an HLA-DRβ polypeptide chain: HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5. The organization of the group of functional genes, as transmitted hereditarily, varies according to individuals, who therefore display different retained haplotypes. If the presence of an HLA-DRB1 gene is still observed, the presence of one or two other genes (e.g., HLA-DRB3, HLA-DRB4, or HLA-DRB5) is optional, depending on the HLA-DRB1 allele carried by the same chromosome. Furthermore, because of the presence of the two haplotypes (one inherited from the mother and the other from the father), the complexity of the mixture of the HLA-DRB sequences to be analyzed is highly variable. Therefore, the analysis of the principal information associated with the HLA-DRB1 alleles may be tricky to interpret, depending on the presence or absence of the other HLA-DRB genes (such as, for example, the HLA-DRB3 gene. Accordingly, it may be advantageous to be able to amplify all of the possible HLA-DRB1 alleles (184 of which have been recorded in the 1997 nomenclature: *Nomenclature for Factors of the HLA System*, 1996; [and] *Tissue Antigens*, Vol. 49, 3–II, March 1997) without

amplifying the HLA-DRB3 alleles that may be present (i.e., 1 or 2 possible alleles from among the 11 alleles recorded in the 1997 nomenclature).

The following two examples illustrate the specific inhibition of the amplification of the DRB3 gene. The first of these examples uses an oligonucleotide that includes a 3' end that has been modified through the incorporation of an aminated arm (oligonucleotide 5858, SEQ ID 3), and the second uses an oligonucleotide that includes a modified 3' end that has an –H and a modified 5' end that includes an acridine (oligonucleotide 5967, SEQ ID 4).

The DNA was extracted in accordance with the traditional methods, consisting of cell lysis, digestion by proteinase K, and then phenolic extraction and purification via precipitation with ethanol. The DNA solutions (whose concentration was adjusted to 100 ng/µl in H_2O) were stored at temperatures of 2 to 8°C.

The following general amplification conditions were employed

– X10 buffer:	10	µl
– Generic 5' primer (5867, SEQ ID No. 1) (10 µM) (0.1 µM, final)	1	µl
– Blocking 5' primer (10 µM) (0 to 1.2 µM, final)	0 to 12	µl
– Generic 3' primer (P2, SEQ ID No. 2) (10 µM) (0.1 µM, final)	1	µl
– dNTP (20 mM) (0.2 mM, final)	1	µl
– Taq polymerase (5 IU/µl) (1.5 U)	0.3	µl
– DNA (100 mg/µl) (100 ng)	1	µl
– H_2O (in sufficient quantity to make up:)	100	µl

The characteristics of the amplification program utilized with Perkin Elmer GeneAmp 9600 equipment are listed below:

- 2 minutes at 95°C (1 cycle)
- 30 seconds at 95°C + 30 seconds at 55°C + 30 seconds at 72°C (32 cycles)
- 7 minutes at 72°C (1 cycle)

The resulting amplification products were examined through the analysis of an aliquot portion (5 µl) via electrophoresis on agarose gel, followed by staining with ethidium bromide. After this examination, the amplicons that were prepared were analyzed using the BioMérieux HLA-DR typing oligodetection kit (Ref. No. 74,500). This test made it possible to determine the HLA-DR typing via a reverse hybridization method using microplates, through the detection and analysis of the HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5) alleles (see PCT patent No. FR 92/00702).

DRB3 blocking with oligo-5'–phosphate / 3'–C_6–NH_2

DNA: OMW line (ECCAC 9058), DRB1*1301, DRB3*01

Generic 5' HLA-DRB primer (oligonucleotide 5867, SEQ ID No. 1): 0.1 µM, final

Generic 3' HLA-DRB primer (oligonucleotide P2, SEQ ID No. 2): 0.1 µM, final

Blocking 5' HLA-DRB3 primer (oligonucleotide 5858, SEQ ID No. 3): 0, 0.3, 0.6, 0.9, 1.2 µM, final

Hybridization on microplates:

The values of the hybridization signals (optical density, as read at a wavelength of 492 nm and multiplied by 1,000), as observed for each of the specific probes, are shown in Table 2 below.

Final concentration of blocking primer 5858 (in µM)	Probe 13	Probe 3 + 6	Probe 52a
0	> 2500	723	928
0.3	2109	578	101
0.6	1901	563	29
0.9	1759	374	31
1.2	1719	503	12
Ratio: 0.6 / 0	0.76	0.78	0.03

Table 2. – Signals indicating the hybridization of blocking primer 5858 with various target sequences, as a function of its concentration.

The calculation of the ratio of the value as read for 0.6 µM, in relation to the value read without blocking, makes it possible to determine the inhibition of the amplification of the DRB3 gene.

The 13 and 3+6 probes are specific for the DRB1 gene, and the 52a probe is specific for the DRB3 gene. The addition of oligonucleotide 5858 during amplification inhibits the amplification of the DRB3 gene, without affecting the amplification of the DRB1 gene. This inhibition is dose dependent. For the DRB3-blocking oligonucleotide, total inhibition is observed at concentrations of 0.6 µM and higher.

Sequencing:

The amplification products (as obtained with or without blocking of the DRB3 gene) were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Ref. 4303152). The electrophoretograms for the reverse reaction are shown below, for the test without blocking and for the test with blocking using oligonucleotide 5858 [at a concentration of] 0.9 µM.

The assembled electrophoretograms for the region of the HLA-DRB genes that codes for amino acids 56 through 65 (in the official HLA nomenclature) illustrate the inhibition of the amplification of the HLA-DRB3 gene (see Figure 9 and Figure 10).

Expected sequence for:	5' > 3'
	56 60 65
DRB1*1301	CCT GAT GCC GAG TAC TGG AAC AGC CAG AAG GAC
DRB3*01	CCT GTC GCC GAG TCC TGG AAC AGC CAG AAG GAC
DRB1*1301 + DRB3*01 (forward)	CCT GWY GCC GAG TMC TGG AAC AGC CAG AAG GAC
DRB1*1301 + DRB3*01 (reverse)	GGA CWR CGG CTC AKG ACC TTG TCG GTC TTC CTG

Sequence read for:	
Test without blocking	GGA CWR CGG CTC AKG ACC TTG TCG GTC TTC CTG
Test with blocking (0.9 µM)	GGA CTA CGG CTC ATG ACC TTG TCG GTC TTC CTG

Thus, the addition of the DRB3-specific blocking primer (oligonucleotide 5858, 3'–C_6–NH_2) inhibits the amplification of the DRB3 gene, as indicated by the disappearance of the related bases at positions 57 and 60, thus demonstrating the absence of the sequence that corresponds to the DRB3*01 allele.

DRB3 blocking with oligo-5'–acridine / 3'–H

DNA: OMW line (ECCAC 9058), DRB1*1301, DRB3*01

Generic 5' HLA-DRB primer (oligonucleotide 5867, SEQ ID No. 1): 0.1 µM, final

Generic 3' HLA-DRB primer (oligonucleotide P2, SEQ ID No. 2): 0.1 µM, final

Blocking 5' HLA-DRB3 primer (oligonucleotide 5967, SEQ ID No. 4): 0, 0.3, 0.6, 0.9, 1.2 µM, final

Hybridization on microplates:

The values of the hybridization signals (optical density, as read at a wavelength of 492 nm and multiplied by 1,000), as observed for each of the specific probes, are shown in Table 3 below.

Final concentration of blocking primer 5967 (in µM)	Probe 13	Probe 3 + 6	Probe 52a
0	2356	907	893
0.3	1227	251	5
0.6	1395	239	0
0.9	799	185	4
1.2	965	161	0
Ratio: 0.6 / 0	0.60	0.30	0

Table 3. – Signals indicating the hybridization of blocking primer 5967 with various target sequences, as a function of its concentration.

The calculation of the ratio of the value as read for 0.6 µM, in relation to the value read without blocking, makes it possible to determine the inhibition of the amplification of the DRB3 gene.

Here again, the addition of oligonucleotide 5967 during amplification inhibits the amplification of the DRB3 gene, without affecting the amplification of the DRB1 gene. This inhibition is dose dependent, and for the DRB3 blocking oligonucleotide, the total inhibition is observed at concentrations of 0.3 µM and higher.

Example 2: Specific blocking of the amplification of the HLA-DRB4 gene

This case is comparable to the one described in Example 1. To simplify the interpretation of the HLA-DRB1 typing, it may be advantageous to limit the HLA-DRB amplification, using the generic HLA-DRB primers, to the DRB1 gene, with no co-amplification of the DRB4 gene. The invention describes the use of DRB4-specific blocking primers.

The experimental protocols are identical to the ones described in Example 1, i.e.:

DNA: T7526 line (ECCAC 9076), DRB1*0901, DRB4*01
Generic 5' HLA-DRB primer (oligonucleotide 5867, SEQ ID No. 1): 0.1 μM, final
Generic 3' HLA-DRB primer (oligonucleotide P2, SEQ ID No. 2): 0.1 μM, final
Blocking 5' HLA-DRB3 primer (oligonucleotide 5965, SEQ ID No. 5): 0, 0.3, 0.6, 0.9, 1.2 μM, final

Hybridization on microplates:
The values of the hybridization signals (optical density, as read at a wavelength of 492 nm and multiplied by 1,000), as observed for each of the specific probes, are shown in Table 4 below.

Final concentration of blocking primer 5965 (in μM)	Probe 9	Probe 53
0	1769	1320
0.3	1935	110
0.6	1754	41
0.9	1750	29
1.2	1516	14
Ratio: 0.6 / 0	0.99	0.03

Table 4. – Signals indicating the hybridization of blocking primer 5965 with various target sequences, as a function of its concentration.

The calculation of the ratio of the value as read for 0.6 μM, in relation to the value read without blocking, makes it possible to determine the inhibition of the amplification of the DRB4 gene.

Probe 9 is specific for the DRB1 gene, and probe 53 is specific for the DRB4 gene. The addition of oligonucleotide 5965 during amplification inhibits the amplification of the DRB4 gene, without however affecting the amplification of the DRB1 gene. This inhibition is dose dependent, and for the DRB3 blocking oligonucleotide, the total inhibition is observed at concentrations of 0.6 μM and higher.

<u>Sequencing</u>:

The amplification products (as obtained with or without blocking of the DRB4 gene) were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Ref. 4303152). The electrophoretograms for the reverse reaction are shown below, for the test without blocking and for the test with blocking using oligonucleotide 5965 [at a concentration of] 0.9 μM.

The assembled electrophoretograms for the region of the HLA-DRB genes that codes for amino acids 29 through 47 (in the official HLA nomenclature) illustrate the inhibition of the amplification of the HLA-DRB4 gene (see Figure 11 and Figure 12).

	5’ > 3’
Expected sequence for:	30 35 40 45
DRB1*0901	AGA GGC ATC TAT AAC CAA GAG GAG AAC GTG CGC TTC GAC ACC GAC GTG GGG GAG TAC
DRB4*01	AGA TAC ATC TAT AAC CAA GAG GAG TAC GCG CGC TAC AAC AGT GAC CTG GGG GAG TAC
DRB1*0901 + DRB4*01 (forward)	AGA KRC ATC TAT AAC CAA GAG GAG WAC GYG CGC TWC RAC AGY GAC STG GGG GAG TAC
DRB1*0901 + DRB4*01 (reverse)	TCT MYG TAG ATA TTG GTT CTC CTC WTG CRC GCG AWG YTG TCR CTG SAC CCC CTC ATG
Sequence read for:	
Test without blocking	TCT MYG TAG ATA TTG GTT CTC CTC WTG CRC GCG AWG YTG TCR CTG SAC CCC CTC ATG
Test with blocking (0.9 µM)	TCT CCG TAG ATA TTG GTT CTC CTC TTG CAC GCG AAG CTG TCG CTG CAC CCC CTC ATG

Thus, the addition of the DRB4-specific blocking primer (oligonucleotide 5965, 3'–C_6–NH_2) inhibits the amplification of the DRB4 gene, as indicated by the disappearance of the related bases at positions 30, 37, 38, 40, 41, 42, and 44, thereby demonstrating the absence of the sequence that corresponds to the DRB4*01 allele.

The present invention can be applied to high-resolution HLA-DRB1 typing, with the specific blocking of the specific amplification of the HLA-DRB3, HLA-DRB4, and HLA-DRB5 genes and of the HLA-DRB2, HLA-DRB6, HLA-DRB7, HLA-DRB8, and HLA-DRB9 pseudogenes. The use of blocking primers reduces the analytical task to a mixture of one (for an homozygotic sample) or two (for an heterozygotic sample) nucleotide sequences.

Such a strategy utilizes HLA-DRB3-specific blocking primers (e.g., oligonucleotides 5816 (SEQ ID No. 6), 5868 (SEQ ID No. 7), and 5885 (SEQ ID No. 8)); HLA-DRB4-specific primers (e.g., oligonucleotides 5883 (SEQ ID No. 9), 5916 (SEQ ID No. 10), and 5917 (SEQ ID No. 11)); and HLA-DRB5-specific primers (e.g., oligonucleotides 5021 (SEQ ID No. 12), 5870 (SEQ ID No. 13), 5871 (SEQ ID No. 14), 5881 (SEQ ID No. 15), 5902 (SEQ ID No. 16), 5903 (SEQ ID No. 17), 5913 (SEQ ID No. 18), and 5914 (SEQ ID No. 19)), modified at their 3' and 5' ends, as described hereinabove.

Complete blocking of the HLA-DRB3, HLA-DRB4, and HLA-DRB5 genes can be achieved through the use of a mixture of blocking primers.

In Table 5 below, "i" stands for inosine.

BioMérieux reference	SEQ ID No.	Nucleotide sequence (5' > 3')	Modified at the 5' end	Modified at the 3' end
5867	1	ATC CTT CGT GTC CCC ACA GCA CG	—	—
P2	2	TCG CCG CTG CTG CAC TGT GAA G	—	—
5858	3	CCC CCC AGC ACG TTT CTT GGA GCT	—	C_6–NH_2
5967	4	CCC CCC AGC ACG TTT CTT GGA GCT	acridine	–H
5965	5	CCC ACA GCA CGT TTC TTG GAG CAi GC	—	C_6–NH_2
5816	6	CCC AGC ACG TTT CTT GGA GCT	—	—
5868	7	CCC CCC AGC ACG TTT CTT GGA GCT	—	—
5885	8	CCC CCC AGC ACG TTT CTT GGA GiT	—	—
5883	9	CAT TTC CTC AAT GGG ACG GAG iiA	—	—
5916	10	CCC CCA GCA CGT TTC TTG GAG CAi GC	—	—
5917	11	CCC ACA GCA CGT TTC TTG GAG CAi GC	—	—
5021	12	CA CGT TTC TTG CAG CAG GA	—	—
5870	13	CA GCA CGT TTC TTG CAG CAG GA	—	—
5871	14	CA iGT TTC TTG CAG CAG GA	—	—
5881	15	CA GCA iGT TTC TTG CAG CAG GA	—	—
5902	16	CCC CCA GCA iGT TTC TTG CAG CAG GA	—	—
5903	17	CCC ACA GCA iGT TTC TTG CAG CAG GA	—	—
5913	18	CCC ACA GCA iGT TTC TTG CAG CAG iA	—	—
5914	19	CCC CCA GCA iGT TTC TTG CAG CAG iA	—	—

Table 5. – Nucleotide sequence of the oligonucleotides used as amplification primers.

Inosine, a non-natural base, is used to fragilize the hybrid consisting of the nucleic acid and the blocking primer. In point of fact, the inosine is linked to its complementary nucleotide by two hydrogen bonds; therefore, when it is substituted for a pyrimidine, the bond between the two strands is weakened in the region of the substitution.

Because one gene can vary from other, related genes by as few as a single base, the bases around this crucial position (on the blocking primer that is complementary to the gene) can advantageously be replaced with inosines. Because the duplex consisting of the nucleic acid and the blocking primer is thereby rendered more fragile, hybridization can take place only if the primer is exactly complementary to the target genetic sequence. Thus, the specificity of the blocking primer is strengthened.

Consequently, the present invention relates to a procedure for the selective amplification of genes that are present in a mixture of related genes, through the use of blocking oligonucleotide primers that correspond to oligonucleotides that include a modified 3' end that does not allow their elongation during the stages consisting of the enzymatic amplification of the target genes.

The invention also relates to the use of blocking primers, in the sense described in Claim 1, that include a modified 5' end that does not allow their displacement or degradation, during the stages consisting of the enzymatic amplification of the target genes by a primer that is specific for a region located closer to the 5' end on the same gene.

When blocking primers are used in the manner described hereinabove, the modification at the 5' end is optional. Thus, two different possibilities are presented.

In a first embodiment, the –OH group at the 3' end is replaced by a group which, of course, is not found in nature, such as (for example) an –H group, a –phosphate group, a –dabcyl group, or a carbon chain terminated by an $-NH_2$ group.

In a second embodiment, the –phosphate group at the 5' end is replaced by a group which, of course, is not found in nature, such as (for example) a –DMT group, an acridine group, a –thiophosphate group, or a so-called "stem-loop" structure.

The blocking primers, as described hereinabove, may also include modifications of the oligonucleotide at a non-terminal position, and may be used to encourage their hybridization with their target sequences.

The blocking primers, as described hereinabove, can hybridize with the coding strand or with the complementary strand (through the use of the 5' blocking primers or the 3' blocking primers).

A blocking primer or a mixture of blocking primers may also be used.

The use of blocking primers is particularly worthwhile in connection with methods for the amplification of target sequences, such as (for example) PCR, TMA, or any other method.

Lastly, the invention relates to the use of one or more blocking primers to inhibit the amplification of the HLA-DRB3, HLA-DRB4, and/or HLA-DRB5, genes, [which primers are] selected from among the ones defined by SEQ ID No. 3 through SEQ ID No. 19 and their complements.

CLAIMS

1. Procedure for the amplification of at least one specific nucleotide sequence of a synthetic or natural nucleic acid contained within a reaction mixture, with the said reaction mixture consisting of at least one nucleic acid that has at least two related nucleotide sequences and/or at least two nucleic acids, each of which includes at least one related nucleotide sequence; the said procedure utilizes at least one type of amplification primer that is capable of hybridizing with nucleic acid in order to allow the amplification of related nucleotide sequences, and is characterized in that the said procedure consists of adding, to the reaction mixture, at least one sequence, which serves as a blocking primer, which is able to:

- Hybridize with at least one nucleotide sequence that is not the specific nucleotide sequence or sequences to be amplified; and

- Inhibit, in its immediate region, the elongation of the amplification trigger.

2. Procedure according to Claim 1, characterized in that the blocking primer or primers are capable of hybridizing with the nucleotide sequence that is not the specific nucleotide sequence to be amplified, or with all of the nucleotide sequences that are not the specific nucleotide sequence to be amplified.

3. Blocking primer utilized in an amplification process, according to either Claim 1 or Claim 2, characterized by the fact that each blocking primer is an oligonucleotide having a base of deoxyribonucleotides and/or ribonucleotides and/or modified nucleotides, such as PNAs or thiophosphate nucleotides.

4. Primer according to Claim 3, characterized by the fact that the each blocking primer includes at least one element that prevents the amplification.

5. Primer according to Claim 4, characterized by the fact that the said element that prevents amplification is located at the 3' end of the blocking primer, and does not allow its elongation.

6. Primer according to Claim 5, characterized by the fact that the said element that prevents amplification is located at the 5' end of the blocking primer and serves as a protective element.

7. Primer according to any one of Claims 4 through 6, characterized by the fact that each element that prevents amplification consists of a nucleotide, a modified nucleotide, or an oligonucleotide that does or does not include at least one modified nucleotide, and by the fact that the said nucleotide, modified nucleotide, or oligonucleotide does not hybridize with the nucleic acid.

8. Primer according to any one of Claims 4 through 6, characterized by the fact that each element that prevents amplification consists of a molecule that is different from a nucleotide or from a modified nucleotide.

9. Primer according to any one of Claims 4 through 7, characterized by the fact that the element consists of at least five, and particularly at least ten, and preferably at least fifteen nucleotides or modified nucleotides, or a mixture of nucleotides and modified nucleotides.

10. Primer according to any one of Claims 4 through 7 or 9, characterized by the fact that the said element is long enough to allow the formation of a loop and of hybridization between the nucleotides and/or the modified nucleotides that constitute the said loop.

11. Primer according to any one of Claims 4 through 7 or 9, characterized by the fact that the said element consists of a so-called "tail" of polynucleotides and/or modified polynucleotides that all include the same bases.

12. Primer in which the element does not allow elongation, according to any one of claims 4, 5, or 7 through 6, characterized by the fact that the said element that is substituted for the hydrogen atom in the hydroxyl group or for the hydroxyl group [itself], located at position 3' on the ribose, is itself located at the 3' end of the nucleic acid.

13. Primer in which the element is protective, according to any one of claims 4 or 6 through 12, characterized by the fact that the said element is:

- Substituted for the phosphate located at position 5' on the ribose, which itself is located at the 5' end of the nucleic acid; or

- Grafted to the phosphate located at position 5' on the ribose, which itself is located at the 5' end of the nucleic acid.

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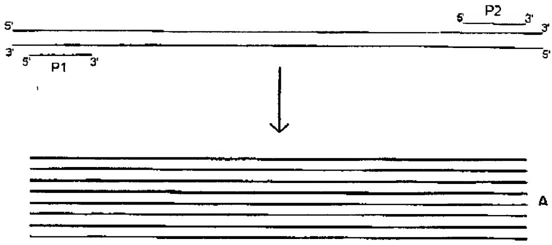

Fig. 1

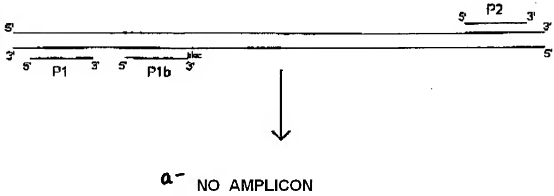

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

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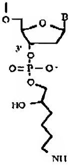
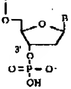
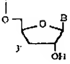


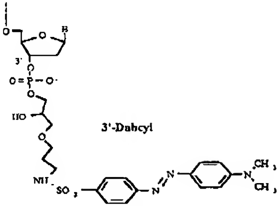
3'-C6-NH2



3'-Phosphate



3'-H



3'-Dabcyl

Fig. 7

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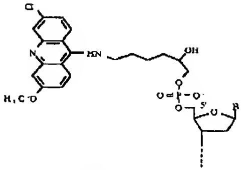
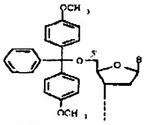
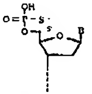


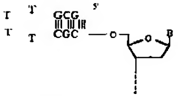
5'-Acridine



5'-DMT



5'-Thiophosphate



5'- Stem-loop

Fig. 8

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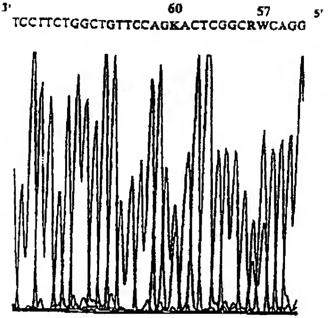

Fig. 9

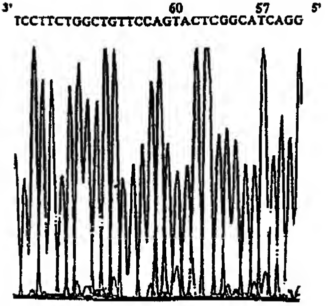

Fig. 10

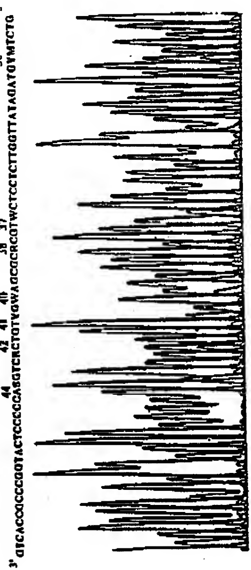

Fig. 11

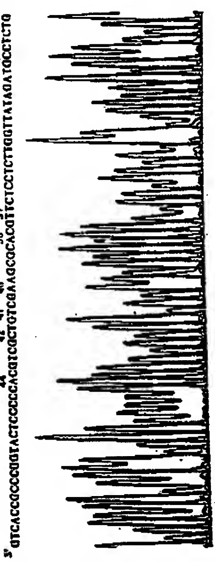

Fig. 12

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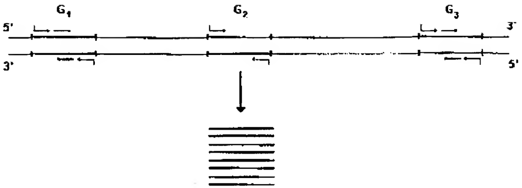

b- Amplification primer

c- Blocking primer

Fig. 13